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The ability of p53 to induce apoptosis requires its sequence-specific DNA binding activity; however, the transactivation deficient p53(gln22-ser23) can still induce apoptosis. Previously, we have shown that the region between residues 22 and 97 in p53 is necessary for such activity. In an effort to more precisely map a domain necessary for apoptosis within the N-terminus, we found that deletion of the N-terminal 23 amino acids compromises, but does not abolish, p53 induction of apoptosis. Surprisingly, p53(Δ1-42), which lacks the N-terminal 42 amino acids and the previously defined activation domain, retains the ability to induce apoptosis to an even higher level than wild-type p53. A more extensive deletion, which eliminates the N-terminal 63 amino acids, renders p53 completely inert in mediating apoptosis. In addition, we found that both p53(Δ1-42) and p53(gln22-ser23) can activate a subset of cellular p53 targets. Furthermore, we showed that residues 53 and 54 are critical for the apoptotic and transcriptional activities of both p53(Δ1-42) and p53(gln22-ser23). Taken together, these data suggest that within residues 43 to 63 lies an apoptotic domain as well as another transcriptional activation domain. We therefore postulate that the apoptotic activity in p53(gln22-ser23) and p53(Δ1-42) is still transcription-dependent.

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INTRODUCTION

The p53 tumor suppressor protein serves as a checkpoint in maintaining genome stability (1-3). Several different biological responses that could play a role in maintaining genome stability have been strongly correlated with wild-type p53 function (1,4). Following stress conditions such as in the presence of damaged DNA or insufficient growth and survival factors, the cellular levels of p53 increase. This leads to one of at least three well understood cellular responses: cell cycle arrest, differentiation, or apoptosis. Several factors have been shown to determine how a cell responds to the accumulation of p53, e.g., cell type and the presence of several cellular and viral proteins (5-9). In addition, the levels of p53 in a given cell can dictate the response of the cell such that lower levels of p53 result in cell cycle arrest (10) or differentiation (11) while higher levels result in apoptosis (10,11).

The functional domains of p53 have been subjected to extensive analysis (1,4,5,10,11). A transcriptional activation domain has been shown to lie within the amino terminal residues 1-42 (12,13). Within this region there are a number of acidic and hydrophobic residues, characteristics of the acidic activator family of transcriptional factors (14). Indeed, a double point mutation of the two hydrophobic amino acids at residues 22 and 23 renders p53 transcriptionally inactive (15). These two residues presumably are required for the interaction of the activation domain with the TATA box binding protein (TBP) and/or TBP-associated factors (TAFs) (16-19).

It is well established that as a transcriptional activator, p53 upregulates p21, a cyclin-dependent kinase inhibitor (20-22), which leads to p53-dependent G1-arrest. However, it is not certain what function(s) of p53 is required for apoptosis. The transactivation function of p53 was shown to be required in some experimental protocols (23-25). There are several candidate genes that play roles in apoptosis that can be activated in response to p53 induction, such as bax (26), IGFBP3 (27), PAG608 (28), Killer/DR5 (29), and several redox-related PIGs genes (30). Several other studies, including our own observations, have provided evidence that p53 might have a transcription-independent function in apoptosis (10,31-33). Recently, the proline-rich region between residues 60 and 90, which comprises five "PXXP" motifs (where P represents proline and X any amino acid), was found to be necessary for efficient growth suppression (34), and apoptosis (35) and to serve as a docking site for transactivation-independent growth arrest induced by Gas1 (36).

Previously, we showed that the region between residues 23 and 97 is necessary for apoptosis (10). To more precisely map such a domain in the N-terminus necessary for apoptosis, we have made several new mutants. By establishing H1299 cell lines that inducibly express these mutants, we found a novel p53 domain that is necessary for mediating apoptosis. Furthermore, we tested these p53 mutants in the human breast cancer cell line, MCF7. We observed several profound differences between MCF7 and H1299 cells in their response to these p53 mutants.

BODY

Experimental procedures

Plasmids and mutagenesis. Mutant p53 cDNAs were generated by PCR using the full-length wild-type p53 cDNA as a template. To generate p53(Δ1-23), the pair of primers used were: forward primer N24, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TAA ACT ACT TCC TGA A; and reverse primer C393, GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT. To generate p53(Δ1-42), the pair of primers used were: forward primer N43, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TTT GAT GCT GTC CCC G; and reverse primer C393. To generate p53(Δ1-63), the pair of primers used were: forward primer N64, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TCC CAG AAT GCC AGA GGC T; and reverse primer C393. To generate p53(gln22-ser23/gln53-ser54), cDNA fragments encoding amino acids 1-59 and 60-393 were amplified independently and ligated together through an internal Ava II site. The p53(gln22-ser23) cDNA was used as a template (15). The pair of primers for the cDNA fragment encoding amino acids 1-59 were: forward primer N1, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC; and reverse primer C59, TTC ATC TGG ACC TGG GTC TTC AGT GCT CTG TTG TTC AAT ATC. The pair of primers for the cDNA fragment encoding amino acids 60-393 were: forward primer N60, ACT GAA GAC CCA GGT CCA; and reverse primer C393. To generate p53(Δ1-42/gln53-ser54), the p53(gln22-ser23/gln53-ser54) cDNA was amplified by forward primer N43 and reverse primer C393. To generate p53(Δ1-42/Δ364-393), the carboxyl terminus of p53(Δ1-42) cDNA beginning at amino acid 144 at the Pvu II site was replaced by the carboxyl terminus of p53(Δ364-393) cDNA (37). Mutations were confirmed by DNA sequencing.

The above mutant p53 cDNAs were cloned separately into a tetracycline-regulated expression vector, 10-3, at its Eco RI site and the resulting plasmids were used to generate cell lines that inducibly express p53.

Cell lines, transfection and selection procedures. The H1299 cell line was purchased from the American Type Culture Collection and grown with Dulbocco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. Transfections were performed using the calcium chloride method as described (38). Cell lines expressing inducible proteins of interest were generated as previously described (10). Individual clones were screened for inducible expression of the p53 protein by Western blot analysis using monoclonal antibodies against p53. The H1299 cell lines that inducibly express either wild-type p53 or p53(Δ364-393) are p53-3 and p53(Δ364-393)-1, respectively, as previously described (9). The H1299 cell line that inducibly expresses p53(Δ62-91) is p53(Δ62-91)-5 (manuscript in preparation).

Western blot analysis. Cells were collected from plates in phosphate-buffered saline (PBS), resuspended with 1× sample buffer, and boiled for 5 min. Western blot analysis was performed as previously described (39). Monoclonal antibodies used to detect p53 were Pab240 and Pab421 (39). The affinity-purified monoclonal antibody against p21 (Ab-1) was purchased from Oncogene Science (Uniondale, NY). Affinity-purified anti-actin polyclonal antibodies was purchased from Sigma (St. Louis, MO).

Growth rate analysis. To determine the rate of cell growth, cells were seeded at 5-10 × 10^4 cells per 60-mm plate, with or without tetracycline (2 μg per ml). The medium was replaced every 72 h. At times indicated, two plates were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted three times by Coulter cell counter. The average number of cells from at least two plates were used for growth rate determination.

FACS analysis. Cells were seeded at 2.0×10^5 per 90-mm plate with or without tetracycline. Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 70 % ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hours. The percentage of cells in sub-G_1, G_0-G_1, S, and G_2-M phases was determined using the ModFit program. The percentage of cells in sub-G_1 phase was used as an index for the degree of apoptosis.

Cell viability assay by trypan blue exclusion. Cells were seeded at 2×10^5 per 90-mm plate with or without tetracycline. Three days after plating, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After stained with trypan blue (Sigma) for 15 min, both live (unstained) and dead (stained) cells were counted two times in a hemocytometer. The percentage of dead cells from control plates was subtracted from the percentage of dead cells from experimental plates and the resulting value was used as an index for the degree of apoptosis.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated using Trizol reagent (BRL-Gibco). Northern blot analysis was performed as described (39). The p21 probe was made from an 1.0-kb Eco RI-Eco RI fragment (20); the mdm-2 probe was made from a 2.1-kb Not I-Sma I fragment (40); the bax probe was made from a 290-bp Pst I-Bgl II fragment (41); the gadd45 probe was from a 400-bp Eco RI-Bam HI fragment (42); the gapdh probe was made from an 1.25-kb Pst I-Pst I cDNA fragment (43); and the MCG14 cDNA probe was a 200-bp PCR fragment identified by CLONTECH PCR-Select cDNA subtraction (manuscript in preparation).

Results

A novel domain within residues 43-63 is necessary for mediating apoptosis. Previously, we showed that p53(Δ1-22), which lacks the N-terminal 22 amino acids, can still induce apoptosis as well as cell cycle arrest (10). Since both residues 22 and 23 are critical for p53 transcriptional activity (15), we decided to determine whether p53(Δ1-23), which deletes the N-terminal 23 amino acids, would also be able to induce apoptosis and activate cellular p53 targets.

We have previously established a cell line that expresses high levels of wild-type p53 called p53-3 (10). This line was established using a tetracycline-regulated expression system as previously described (44). Using similar techniques, we established nine stable cell lines that express p53(Δ1-23). Three representative cell lines, p53(Δ1-23)-9, -10 and -23, are shown in Fig. 1A. Western blot analysis showed that these cell lines express p53(Δ1-23) at levels comparable to wild-type p53 in p53-3 cells (Fig. 1A). To characterize p53(Δ1-23), we looked at

its transcriptional and apoptotic activities and the growth rate of the cell line p53(Δ1-23)-9. The transcriptional activity was determined by monitoring the expression of the endogenous gene, p21, a well defined transcriptional target of p53 (20). We found that p53(Δ1-23) is still capable of activating p21, albeit to a much less degree than wild-type p53 (Fig. 1A). Next, the growth rates of p53(Δ1-23)-9 cells under both uninduced and induced conditions were determined, and these cells failed to multiply following p53 expression (Fig. 1B). To exclude potential effects of the regulator tetracycline and/or the tet-vp16 transactivator (44) on cell growth, we analyzed the growth rate of the cell line H24-1, which was similarly established but did not express any protein. The results showed that the growth rates of H24-1 cells under both the uninduced (+ tet) and induced (-tet) conditions were nearly identical (Fig. 1C), indicating that both tetracycline and tet-vp16 transactivator have no effect on cell growth. It is well established that the percentage of cells containing a sub-G_1 DNA content reflects the extent to which cells are undergoing apoptosis (10,24,32,44). Since p53 can induce apoptosis in H1299 cells (9, 31), FACS analysis was used to observe the extent of apoptosis by determining the distribution of cells in each phase of the cell cycle. The results showed that 18% of cells expressing p53(Δ1-23) had a sub-G_1 DNA content 3 days after induction of this mutant, compared to less than 5% of the same cells expressing no p53 (Fig. 1D and 1E; Table 1). Trypan blue exclusion assay showed that 15 % of cells were dead, which is consistent with FACS analysis. In contrast, about 45% and 30% of cells had a sub-G1 DNA content at day 3 following expression of either wild-type p53 or transactivation deficient p53(gln22-ser23), respectively (Table 1). The FACS results also showed that the number of cells in S phase was decreased from 38% to 22.3 % following induction of p53(Δ1-23) and these cells primarily arrested in G_1 (Fig. 1D and 1E). Similar results were obtained using another high p53(Δ1-23) producer, p53(Δ1-23)-10.

Since p53(Δ1-23) is still capable of inducing apoptosis and p53 activation domain lies within residues 1-42 (12,13), we determined whether the other half (residues 24 to 42) of the previously defined activation domain is required for apoptosis. To this end, we established 16 individual stable cell lines that inducibly express p53(Δ1-42) that lacks the N-terminal 42 amino acids. Three representative cell lines, p53(Δ1-42)-2, -5. and -11, were shown in Fig. 2A. Consistent with previous results that p53(gln22-ser23) cannot activate p21 (10,23,34,45), p53(Δ1-42) only minimally activated p21 as compared to wild-type p53 (Fig. 2A). We then determined the growth rate of a high producer, p53(Δ1-42)-2. Surprisingly, we found that a majority of cells died within 3 days following induction of p53(Δ1-42) (Fig. 2B). In addition, both trypan blue exclusion assay and FACS analysis showed that approximately 50-68% of cells underwent apoptosis (Fig. 2C; table 1). Similar results were obtained from several other cell lines. These results suggest that the entire previously defined activation domain within the N-terminal 42 amino acids is dispensable for apoptosis. In fact, deletion of this region enhanced the ability of p53 to induce apoptosis (Table 1).

To further delineate the domain in the N-terminus required for apoptosis, we generated seven inducible cell lines expressing p53(Δ1-63) which lacks the N-terminal 63 amino acids but contains an intact proline-rich region. Three representative cell lines, p53(Δ1-63)-14, -22 and -27, were shown in Fig. 3A, and the activity of p53(Δ1-63) was analyzed as above. The results showed that p53(Δ1-63) was unable to activate p21 expression (Fig. 3A), and p53(Δ1-63)-14 cells, a high p53 producer, continued to multiply when p53(Δ1-63) was induced (Fig. 3B).

Furthermore, both FACS analysis and trypan blue exclusion assay showed that neither apoptosis nor cell cycle arrest were observed in cells expressing p53(Δ1-63) (Fig. 3C and Table 1).

Within residues 43-63 lies another activation domain that overlaps with the domain necessary for mediating apoptosis. The ability of transactivation deficient p53(gln22-ser23) to induce apoptosis leads to the hypothesis that p53 has transcription-independent apoptotic activity (10,32,33). Since p53(Δ1-42) lacks the previously defined activation domain and only minimally activates p21 as determined by Western blot analysis (Fig. 2A), it appears that it can induce apoptosis in a transcription-independent manner. To ascertain whether p53(Δ1-42) contains a transcriptional activity, the expression patterns of four well-defined cellular p53 targets, p21, mdm2, gadd45 and bax, were analyzed in cells expressing p53(Δ1-42) by Northern blot analysis (Fig. 4A). The expression levels of these genes in cells with or without p53 were quantitated by PhosphorImage scanner and the fold increase of their relative mRNAs was calculated after normalization to gapdh mRNA levels (Table 2). The results showed clearly that p53(Δ1-42) significantly activated mdm2 (8 fold), gadd45 (7.03 fold) and bax (3.9 fold) but only minimally activated p21 (1.83 fold). As expected, wild-type p53 but not mutant p53(gln22-ser23) activated these cellular p53 targets (Fig. 4A; table 2). As a control, p53(Δ64-91), which lacks all of the five PXXP motifs, was examined. The proline-rich domain in p53 is dispensable for transactivation (34,35). As expected, p53(Δ64-91) activated these p53 targets (Fig. 4A and Table 2). Since p53(Δ1-63) failed to activate any of these p53-regulated genes (data not shown), the results suggest that another activation domain lies within residues 43-63. For clarity, we designate the originally defined activation domain located within residues 1-42 as activation domain I and this novel domain as activation domain II.

The above observations raise the following question: why does p53(gln22-ser23) fail to activate these well-defined p53 transcriptional targets (Fig. 4A; Table 2) despite the fact that it still contains an intact activation domain II? One of the possibilities is that p53(gln22-ser23) might be still able to activate a subset of p53 transcriptional targets which have yet been identified. To this end, we tested the expression patterns of several potential p53 targets identified in our laboratory. We found that one putative p53 transcriptional target, MCG14, was activated by p53(gln22-ser23) to a level comparable to that by wild-type p53, p53(Δ1-42), p53(Δ64-91), and p53(Δ364-393) (Fig. 4B).

Since a double point mutation at residues 22 and 23 abolishes the transcriptional activity of the activation domain I (15), we looked for analogous hydrophobic amino acids within the activation domain II. Two were found: tryptophan at residue 53 and phenylanaline at residue 54. We therefore made identical mutations in these two amino acids in p53(gln22-ser23) or p53(Δ1-42), changing tryptophan 53 to glutamine and phenylanaline 54 to serine to generate p53(gln22-ser23/gln53-ser54) and p53(Δ1-42/gln53-ser54). We then established a number of cell lines that inducibly express these mutants, and their ability to induce apoptosis and activate cellular p53 targets were similarly analyzed as above. Three representative cell lines that express either p53(gln22-ser23/gln53-ser54) or p53(Δ1-42/gln53-ser54) are shown in Fig. 5A and 5C, respectively. As expected, Western blot analysis showed that p21 was not activated by either of these mutants (Fig. 5A and 5C, bottom panel). In addition, these mutants were unable to induce apoptosis, as demonstrated by the rate of cell growth (Fig. 5B and 5D), trypan blue exclusion assay and FACS analysis (Table 1). Furthermore, the putative cellular p53 target MCG14, which can be activated by p53(Δ1-42) and p53(gln22-ser23) (Fig. 4B), failed to be activated in cells expressing either p53(gln22-ser23/gln53-ser54) or p53(Δ1-42/gln53-ser54) (Fig. 4C). These

results indicate that residues 53 and 54 are critical for the novel domain within residues 43-63 to induce apoptosis and activate cellular p53 targets.

The C-terminal 30 amino acids are dispensable for p53(Δ1-42) to induce apoptosis. In an effort to define a minimum region in p53 required for apoptosis (Aim 3), we showed above that deletion of the previously defined activation domain (activation domain I) located within the N-terminal 42 amino acids slightly enhanced the apoptotic activity of p53. Previously, we showed that deletion of the C-terminal 30 amino acids severely compromises the ability of p53 to induce apoptosis (10). To determine whether the C-terminal regulatory domain is necessary for p53(Δ1-42) to induce apoptosis, we generated a mutant, p53(Δ1-42/Δ364-393) which lacks both the activation domain I and the C-terminal regulatory domain. Several H1299 cell lines that inducibly express such mutant were established (Fig. 6A; top panel). Interestingly, we found that while p53(Δ1-42) has a minimal activity in inducing p21 (Fig 2A; top panel), p21 was substantially activated by p53(Δ1-42/Δ364-393) (Fig. 6A; bottom panel). In addition, both growth rate and FACS analyses showed that p53(Δ1-42/Δ364-393)-expressing cells failed to grow (Fig. 6B) and arrested primarily in G_1 (Fig. 6C). Furthermore, approximately 50% of cells expressing such mutant underwent apoptosis as compared with only 5% of control cells (data not shown). Further characterization of this mutant is in progress. Nevertheless, the C-terminal 30 residues are dispensable when the activation domain I is deleted.

MCF7 breast cancer cells are more resistant to p53-mediated apoptosis than H1299 lung cancer cells. To determine the mechanism of p53-mediated apoptosis in breast cancer cells, we generated several MCF7 cell lines that inducibly express wild-type p53 (lanes 1-4; M7-p53-6 and -19) and p53(Δ1-42/Δ364-393) (lanes 9-12; M7-p53(Δ1-42/Δ364-393)-22 and -16), respectively (Fig. 7A). MCF7 cells carry an endogenous wild-type p53 gene. However, without DNA damage, p53 is expressed at such low level that is undetectable by Western blot analysis (Fig. 7A, lanes 1, 3, 9, and 11). Upon withdrawal of tetracycline from the culture medium, wild-type p53 and p53(Δ1-42/Δ364-393) were induced (lanes 2, 4, 10, and 12). H24-p53(Δ1-42)-2 (as described in Fig. 2) and H24-(Δ1-42/Δ364-393)-7 (as described in Fig. 6) are H1299 cells that inducibly express p53(Δ1-42) and p53(Δ1-42/Δ364-393), respectively, both of which underwent apoptosis following p53 expression. To determine the activity of p53 in MCF7 cells, we analyzed the growth rates of M7-p53-6 and M7-p53(Δ1-42/Δ364-393)-16 cells (Fig. 7B and 7C). Surprisingly, we found that while p53(Δ1-42/Δ364-393) somewhat inhibited cell growth, the amount of wild-type p53 in M7-p53-6 cells has no effect on cell growth.

Recommendations in relation to the Statement of Work

Tasks 1-2: We have tried several times to express either wild-type p53 or various mutated forms of p53 in MDA-MB-453 or -157 cells. We found that these cells simply failed to grow (maybe become senescent) upon prolonged passages in vitro. Thus, we chose MCF7 cells and have established several cell lines (Fig. 7). We will continue to use MCF7 cells to generate additional cell lines to analyze p53-mediated apoptosis.

Tasks 3-5: In progress.

Task 6: A number of short deletion and point mutations of p53 have been generated as described in this report.

Task 7: We identified a novel domain and a minimal region of p53 for apoptosis as described in this report.

Task 8: Currently, we analyze a p53-interacting protein, p73. In progress.

Tasks 9-10: To be done.

CONCLUSION

A novel p53 functional domain

The p53 protein has been divided into several functional domains (1,4,5): (a) an activation domain which lies within residues 1-42 that has been shown to be required for both transcriptional activation and repression (12,13,15,46); (b) a newly identified proline-rich domain within residues 64-91 which is necessary for efficient growth suppression (34), apoptosis (35), and for mediating gas1-dependent growth arrest (36); (c) a sequence-specific DNA binding domain which lies within the central, conserved portion of the protein (1,4); (d) a nuclear localization signal which lies within residues 316-325 (1,4); (e) a tetramerization domain which lies within 334-356 (1,4); and (f) a C-terminal basic domain which binds DNA non-specifically and regulates the sequence-specific DNA binding activity (1,4).

Here we found that within residues 43-63 lies another novel domain that is necessary for apoptosis on the basis of following observations: (i) p53(Δ1-42), which lacks the N-terminal 42 amino acids and the previously defined activation domain, contains a strong apoptotic activity; (ii) p53(Δ1-63), which lacks the N-terminal 63 amino acids but contains intact PXXP motifs, has no apoptotic activity; (iii) a double point mutation at residues 53 and 54 renders both p53(Δ1-42/gln53-ser54) and p53(gln22-ser23/gln53-ser54) completely inert in inducing apoptosis; and (iv) codon 53 is one of the frequently mutated sites outside the DNA binding domain in the p53 gene in human tumors (47), which underscores the importance of the apoptotic function within residues 43-63 in p53 tumor suppression.

How does this novel domain mediate an apoptotic activity? Previously, it was shown that p53(gln22-ser23), which cannot activate several cellular p53 targets (10,15,23,32,45), is still capable of inducing apoptosis (10,32,35,48), and a p53 mutant, which lacks the proline-rich region, is capable of activating several p53 targets (34,35) but cannot induce apoptosis (34,35). These results lead to a hypothesis that p53 has both transcription-dependent and -independent functions in apoptosis. However, it is well established that p53 mutants that are defective in sequence-specific DNA binding activity are also inert in inducing apoptosis (1,4,5), suggesting that p53 sequence-specific DNA binding activity and possibly its sequence-specific transcriptional activity are required for inducing apoptosis. Here we found that p53(Δ1-42), which lacks the entire previously defined activation domain I, not only induces apoptosis, but also activates the mdm2, bax, and gadd45 genes through its activation domain II located between residues 43-63 (Fig. 4; table 2). Since p53(gln22-ser23) contains an intact activation domain II, we hypothesized that it might still contain transcriptional activity. Indeed, we found that p53(gln22-ser23) can activate one putative p53 targets, MCG14. Furthermore, a double point mutation at residues 53 and 54 completely abolishes the ability of both p53(gln22-ser23/gln53-ser54) and p53(Δ1-42/gln53-ser54) to activate MCG14 and induce apoptosis. Consistent with our results, Candau et al. (49) recently showed that within residues 40-83 lies a sub-activation domain, which can activate a reporter gene under control of a promoter with a p53 responsive element when p53 is cotransfected, and a double point mutation at residues 53 and 54 also abolished the transcriptional activity of the sub-activation domain. These results suggest that p53 has two independent activation domains. A second activation domain within a transcription factor is not without precedent. Herpes simplex virus protein VP16 also contains two independent activation domains (50). Thus, it appears that in response to various stress conditions and their subsequent modifications, the two independent activation domains might serve as an intrinsic factor of p53 that determines whether a given p53 target is activated. While bax, mdm2, and gadd45 are the activation domain II-regulated gene products, these cellular p53 targets might not mediate the p53-dependent apoptosis on the basis of two observations: (i) these genes were not activated by

p53(gln22-ser23) which is competent in inducing apoptosis (Fig. 4A; table 2); (ii) these genes were activated by p53(Δ62-91) which is defective in inducing apoptosis (Fig. 4A; table 2). Since cell type has been shown to influence the cellular response (cell cycle arrest or apoptosis) to p53 (4,5,9), cellular genetic background might then determine the modification of the two activation domains. Therefore, the results obtained in H1299 cells need to be confirmed in other cell types.

It is intriguing that although p53(gln22-ser23) contains an intact activation domain II, it fails to activate bax, gadd45, and mdm2 (Fig. 4A). Since both p53(Δ1-23) and p53(Δ1-42) can activate these p53 targets, it suggests that the presence of the first 23 amino acids may mask the ability of the activation domain II in p53(gln22-ser23) to activate these cellular p53 targets. Alternatively, it is also possible that when the activation domain I is inactivated by a double point mutation at residues 22 and 23, the N-terminal 42 residues might then inhibit or block interaction of a co-activator (or an adaptor) with the activation domain II that is required for activation of some p53 targets, such as mdm2, p21, bax, and gadd45, but not for activation of other p53 targets, such as MCG14. It is important to note that although the activation domain I is primarily responsible for activation of p21, the level of p21 in cells expressing either p53(Δ1-23) and p53(Δ1-42) was slightly increased upon p53 induction (Fig. 1A and 2A), suggesting that the activation domain II can weakly activate p21. Furthermore, our preliminary studies showed that activation of p21 was compromised by a double point mutation at residues 53 and 54 when p53(gln53-ser54) was expressed at a low to intermediate level (unpublished results), consistent with the idea that the activation domain II contributes to the activation of p21. Since several clones that express various expression levels of the target genes are required for determining the function of the targets (51), these results remain to be confirmed.

Previously, it was shown that overexpression of p21 can protect human colorectal carcinoma RKO cells from prostaglandin A2-mediated apoptosis (52). Lack of p21 expression due to homologous deletion of the p21 gene also renders HCT116 colorectal cancer cells susceptible to apoptosis following treatment with either γ-radiation or chemotherapeutic agents (53). In addition, a significant fraction of tumors in mice deriving from $p21^{-/-}$ HCT116 cancer cells were completely cured while all tumors deriving from $p21^{+/+}$ cancer cells underwent regrowth after treatment with γ-radiation (53). It is interesting to note that p53(gln22-ser23) and p53(Δ1-42), both of which lack a functional activation domain I, cannot significantly activate p21 (Fig. 2 and 4; table 2), but can induce apoptosis (table 1). The strong apoptotic activity conferred by p53(Δ1-42) might be due to its failure of activating p21. Thus, we have generated a mutant, p53(Δ1-42), that might be better than wild-type p53 in the elimination of cancer cells and therefore a potential candidate for gene therapy.

A minimal region in p53 that is required for mediating apoptosis

As shown in Fig. 6, p53(Δ1-42/Δ364-393) is as competent as wild-type p53 and p53(Δ1-42) in inducing apoptosis and cell cycle arrest. Thus, we have generated a smallest active p53 molecule so far available. It has been shown that both N- and C-termini in p53 can be modified, such as phosphorylation, and interaction of MDM2 with the N-terminal activation domain I interferes p53 activity. The new p53 deletion mutant is not subject to the negative regulation of MDM2 and the C-terminal regulatory domain. Therefore, such mutant may even be better than both wild-type p53 and p53(Δ1-42) as an agent in gene therapy.

Activity of p53 in MCF7 breast cancer cells

Our preliminary evidence suggest that the levels of p53 expressed in MCF7 cells are not sufficient to induce cell cycle arrest and apoptosis, but it is also possible that MCF7 cells are more resistant to p53-mediated apoptosis than H1299 lung cancer cells. Additional experiments are in progress to determine whether p21 and other cellular p53 targets are activated by the inducible wild-type p53 and p53(Δ1-42/Δ364-393) in MCF7 cells. Furthermore, we will generate additional high p53-producing MCF7 cell lines by cloning the lamin 5' untranslated region (UTR) upstream of p53 cDNA in the 10-3 tetracycline-inducible expression vector. Our preliminary results have shown that the lamin 5' UTR substantially enhances p53 expression in our inducible expression system. Nevertheless, cell lines that we have already generated may be ideal to analyze a cooperative induction of apoptosis between exogenous inducible p53 and endogenous wild-type p53 following DNA damage (Task 3).

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APPENDIX A: Figure Legends

Fig. 1. The N-terminal 23 amino acids are dispensable for apoptosis. (A) Levels of p53, p21 and actin in p53-3, and p53(Δ1-23)-9, -10 and -23 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (-) or cells induced to express (+) wild-type p53 or p53(Δ1-23). The upper portion of the blot was probed with a mixture of p53 monoclonal antibodies Pab421 and PAb240 and actin polyclonal antibody. Mutant p53(Δ1-23) migrates faster than wild-type p53 because it is missing 23 amino acids. The lower portion of the blot was probed with p21 monoclonal antibody. (B) Growth rates of p53(Δ1-23)-9 cells in the presence (◇) or absence (□) of p53 were measured as described in Materials and Methods. (C) Growth rates of H24-1 cells in the presence (□) or absence (◇) of tetracycline. (D) DNA contents were quantitated by propidium iodide staining of fixed cells at day 3 following withdrawal of tetracycline as described in Materials and Methods. (E) The percentages of p53(Δ1-23)-9 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days were quantitated using Modfit program as described in Materials and Methods.

Fig. 2. p53(Δ1-42), which lacks the previously defined activation domain, can mediate apoptosis. (A) Levels of p53, p21 and actin in p53-3, and p53(Δ1-42)-2, -5 and -11 cell lines were assayed by Western blot analysis. (B) Growth rates of p53(Δ1-42)-2 cells in the presence (◇) or absence (□) of p53. (C) The percentages of p53(Δ1-42)-2 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those in Fig. 1.

Fig. 3. p53(Δ1-63), which lacks the N-terminal 63 amino acids but contains intact PXXP motifs, failed to induce apoptosis. (A) Levels of p53, p21 and actin in p53-3, and p53(Δ1-63)-14, -22 and -27 cell lines were assayed by Western blot analysis. (B) Growth rates of p53(Δ1-63)-14 cells in the presence (◇) or absence (□) of p53. (C) The percentages of p53(Δ1-63)-14 cells in sub-G_1, G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those in Fig. 1.

Fig. 4. Within residues 43-63 lies another activation domain. (A) Northern blots were prepared using 10 μg of total RNA isolated from uninduced cells (-) or cells induced to express (+) wild-type p53, p53(Δ1-42), p53(Δ62-91), or p53(gln22-ser23). The blots were probed with p21, mdm2, gadd45, bax, and gapdh cDNAs, respectively. (B) A Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (-) or cells induced to express (+) wild-type p53, p53(Δ1-42), p53(Δ62-91), p53(Δ364-393), or p53(gln22-ser23). The blot was probed with MCG14 cDNA. (C) A Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (-) or cells induced to express (+) wild-type p53, p53(gln22-ser23/gln53-ser54), or p53(Δ1-42/gln53-ser54). The blot was probed with MCG14 cDNA.

Fig. 5. A double point mutation at residues 53 and 54 renders both p53(gln22-ser23/gln53-ser54) and p53(Δ1-42/gln53-ser54) completely inert in inducing apoptosis. (A) Levels of p53, p21 and actin in p53-3, and p53(gln22-ser23/gln53-ser54)-9, -11, and -12 cell lines were assayed by Western blot analysis. (B) Growth rates of

p53(gln22-ser23/gln53-ser54)-9 cells in the presence (◇) or absence (□) of p53. (C) Levels of p53, p21 and actin in p53-3, and p53(Δ1-42/gln53-ser54)-1, -9, and -11 cell lines were assayed by Western blot analysis. (D) Growth rates of p53(Δ1-42/gln53-ser54)-11 cells in the presence (◇) or absence (□) of p53. The experiments were performed in an identical manner to those in Fig. 1.

Fig. 6. The C-terminal 30 amino acids are dispensable for p53(Δ1-42) to induce apoptosis. (A) Levels of p53 and p21 in p53-3, and p53(Δ1-42/Δ364-393)-1, 3, 5, 6, and 7 cell lines were assayed by Western blot analysis. (B) Growth rates of p53(Δ1-42/Δ364-393)-7 cells in the presence (◇) or absence (□) of p53. (C) The percentages of p53(Δ1-42/Δ364-393)-7 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those in Fig. 1.

Fig. 7. MCF7 breast cancer cells are more resistant to p53-mediated apoptosis than H1299 lung cancer cells. (A) Levels of p53 in p53-3, M7-p53-6 and -19, H24-p53(Δ1-42)-2, H24-p53(Δ1-42/Δ364-393)-7, M7-p53(Δ1-42/Δ364-393)-7, 22, and 16 cell lines were assayed by Western blot analysis. (B) Growth rates of M7-p53-6 cells in the presence (◇) or absence (□) of p53. (C) M7-p53(Δ1-42/Δ364-393)-16 cells in the presence (◇) or absence (□) of p53. The experiments were performed in an identical manner to those in Fig. 1.

APPENDIX B: Table 1

Table 1. Characteristics of various mutant p53 proteins

	Arrest [a]	Apoptosis [b]
wild-type p53	+++	45
p53(gln22-ser23)	-	30
p53(gln22-ser23/gln53-ser54)	-	-
p53(Δ1-22)	+++	>60
p53(Δ1-23)	++	15-18
p53(Δ1-42)	+/-	50-68
p53(Δ1-42/gln53-ser54)	-	-
p53(Δ1-63)	-	-

(a) Arrest was assayed by the relative growth rate of cells and the number of cells in S phase.
(b) Apoptosis was assayed at day 3 by the percentage of cells staining with trypan blue and by determination of the sub-G_1 phase cells using the Modfit program.

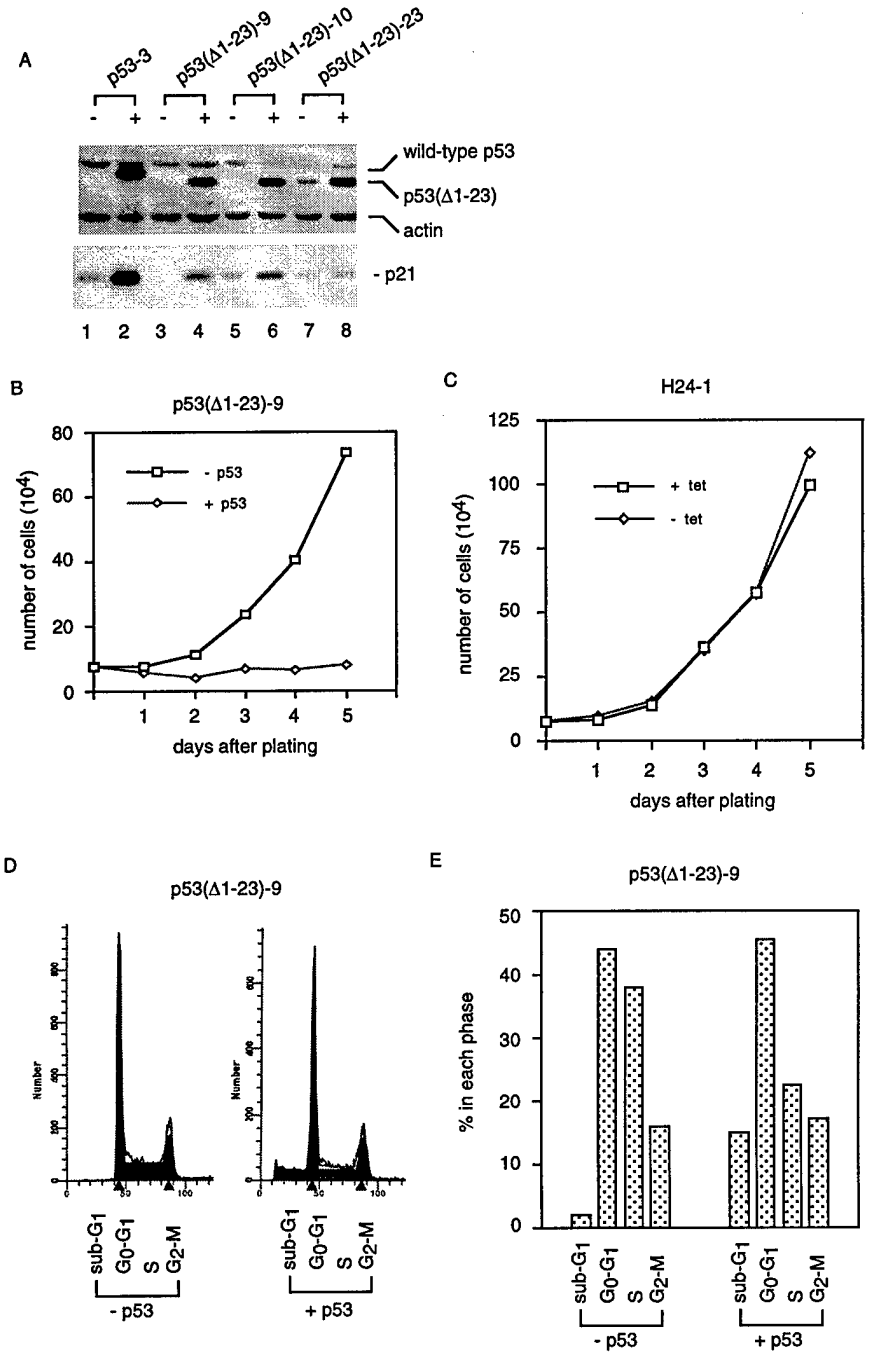
APPENDIX B: Table 2

Table 2. Transcriptional activities of various mutant p53 proteins

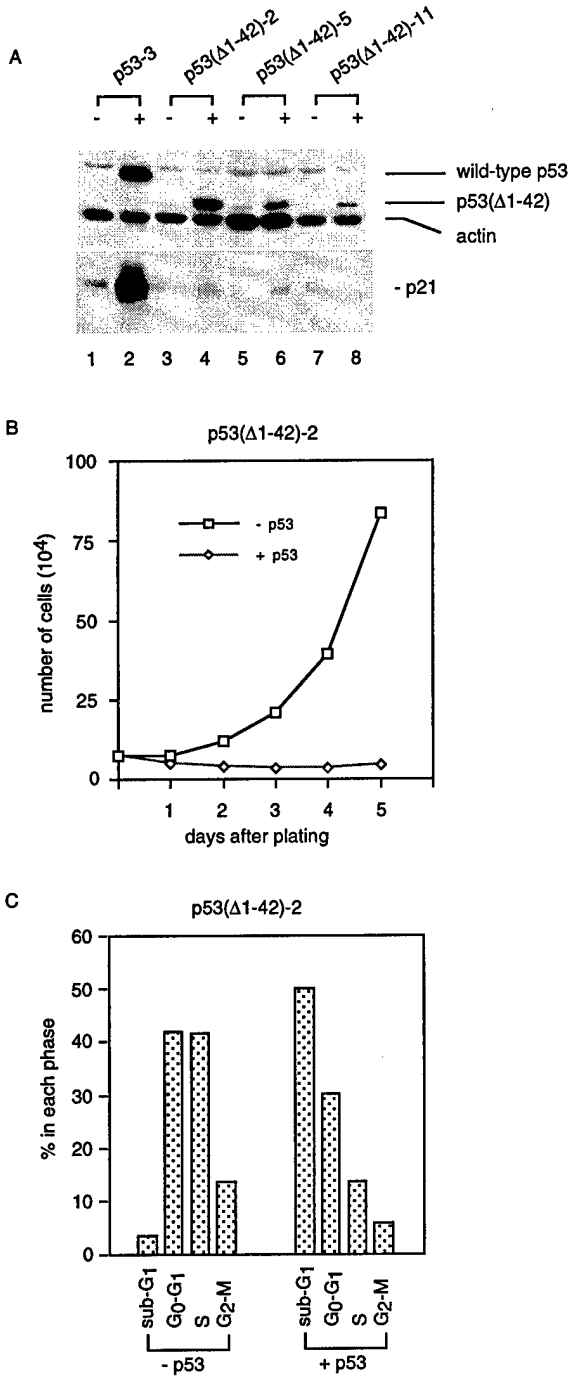
	Fold increase in relative mRNA [a]			
	p21	mdm2	gadd45	bax
Wild-type p53	6.79	40.6	9.45	4.2
p53(Δ1-42)	1.83	8.8	7.03	3.9
p53(Δ62-91)	3.65	6	7	2.7
p53(gln22-ser23)	1.43	1.2	1.44	1.3

[a] Fold = mRNA(+p53)/mRNA(-p53)

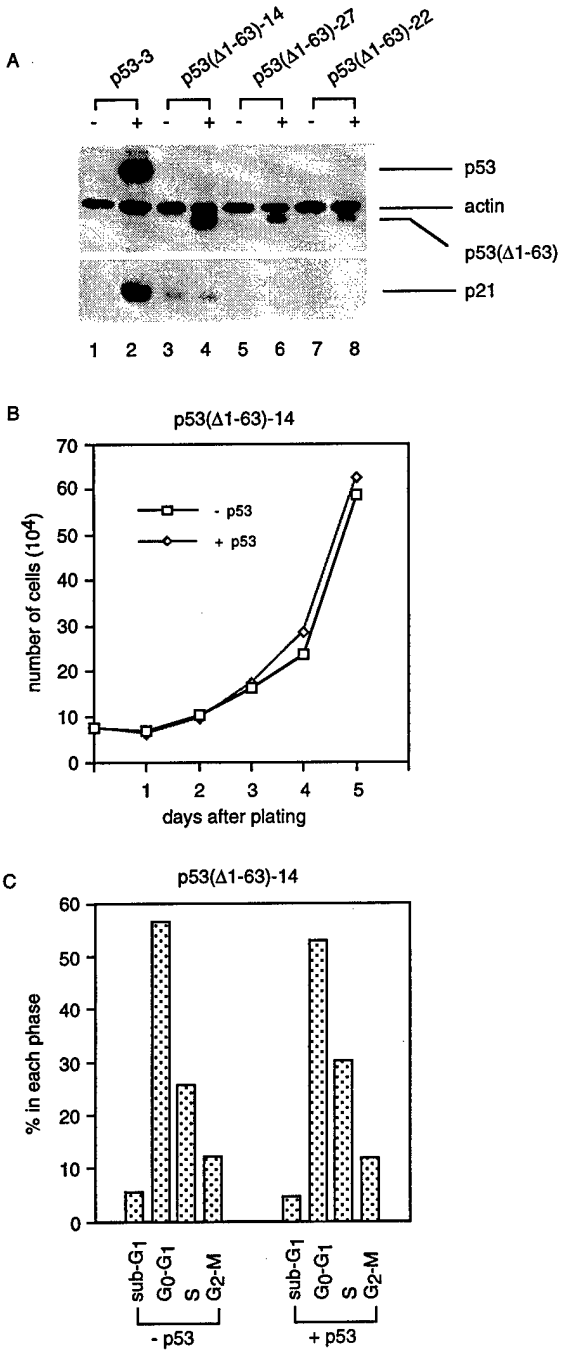
APPENDIX C: Figure 1



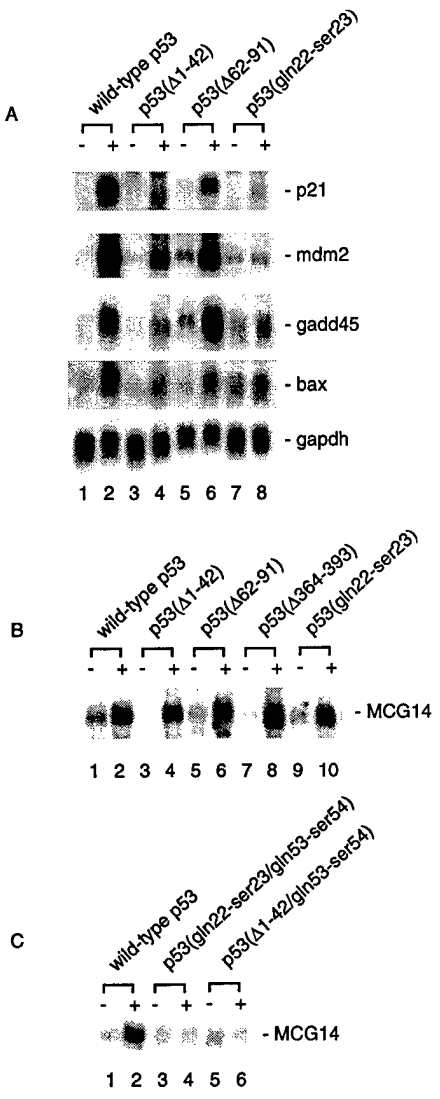
APPENDIX C: Figure 2



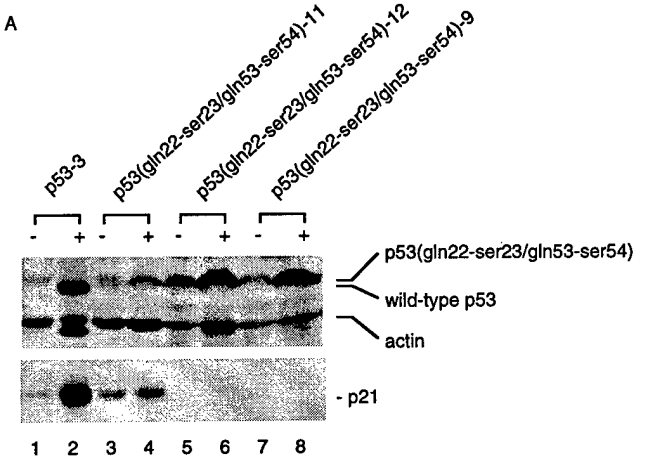
APPENDIX C: Figure 3

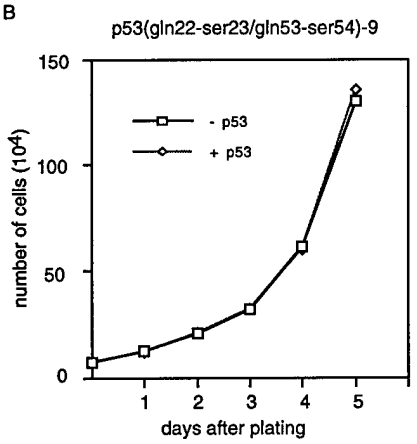


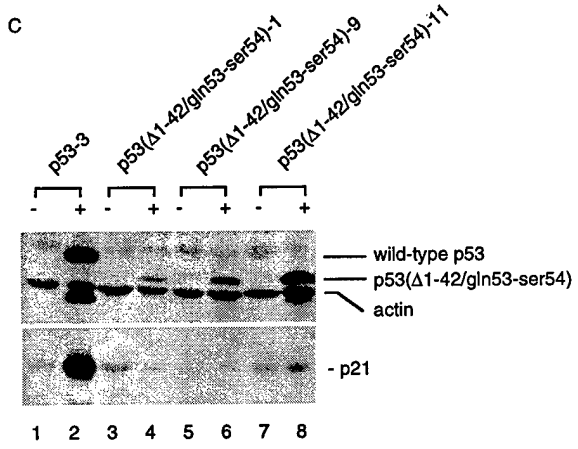
APPENDIX C: Figure 4

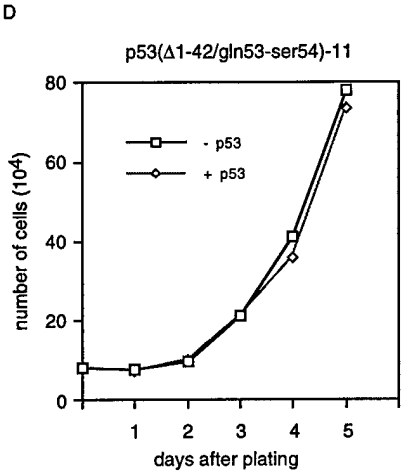


APPENDIX C: Figure 5

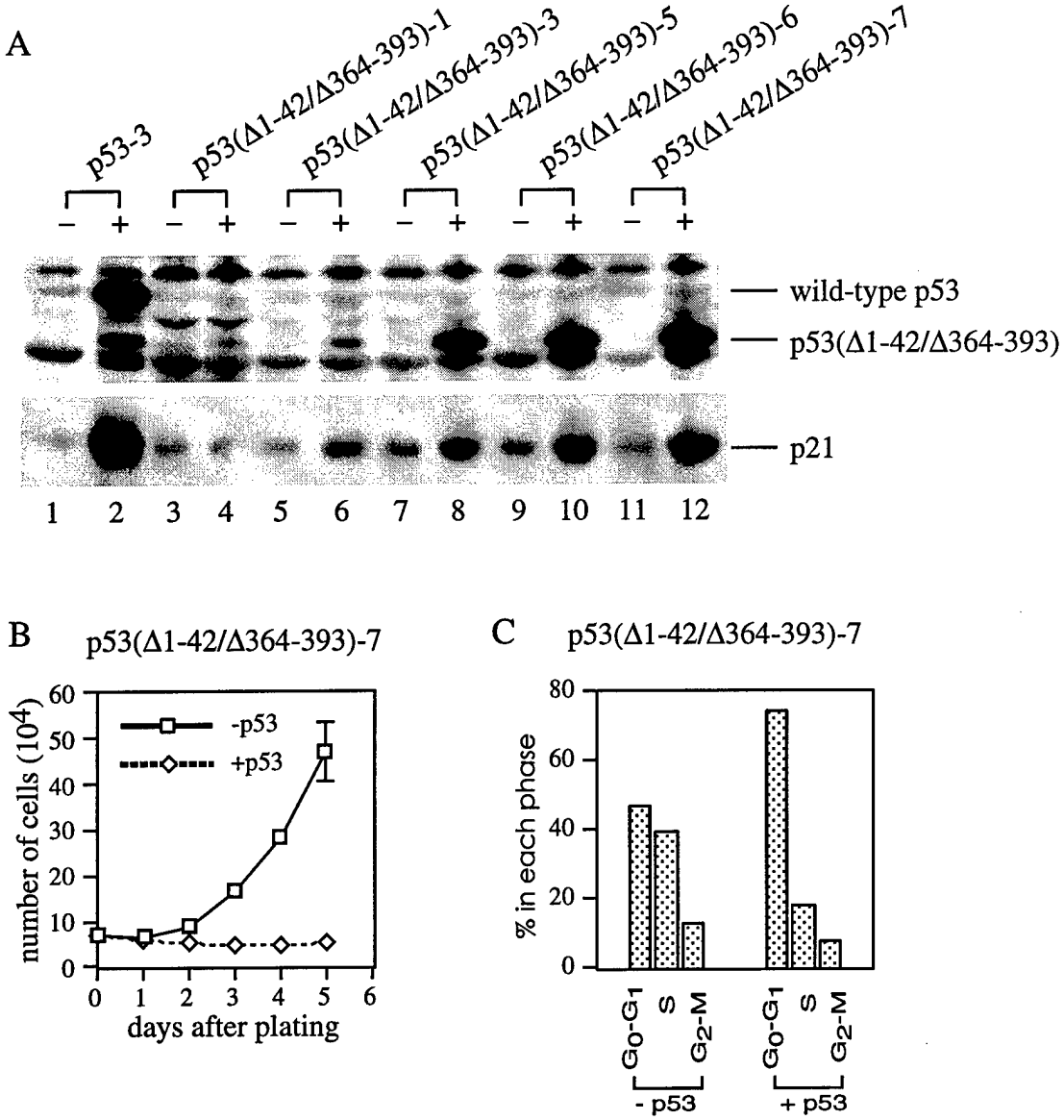




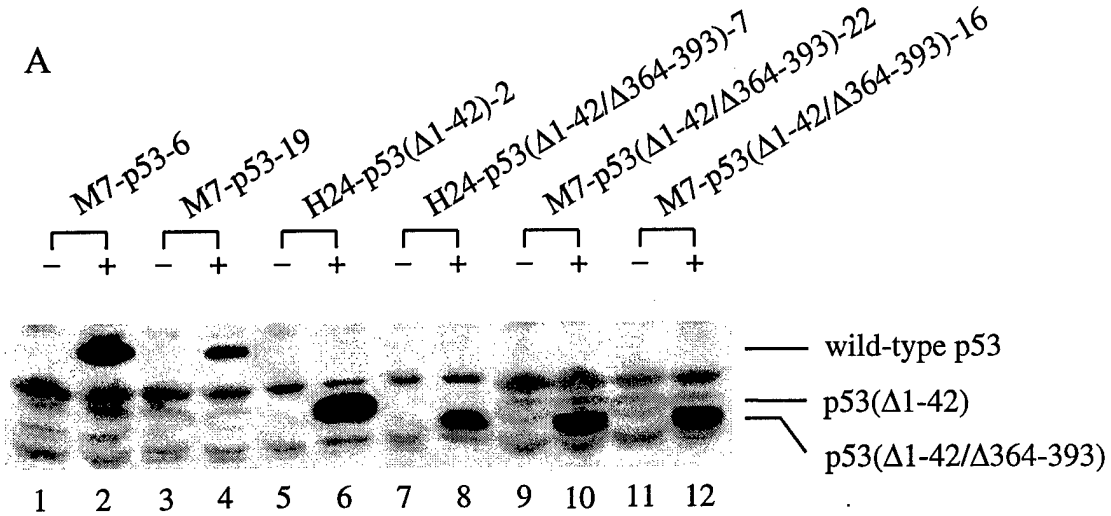


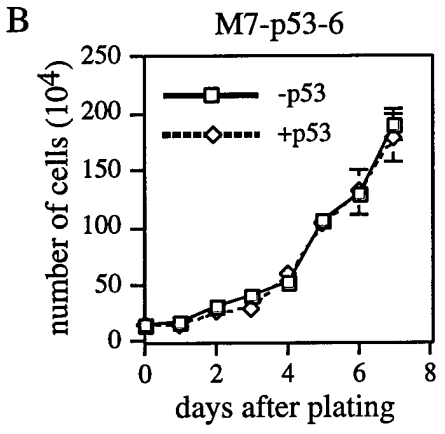


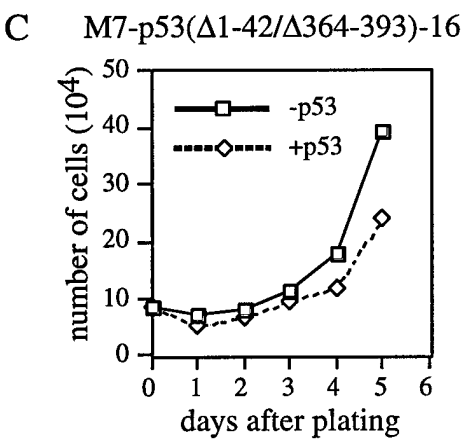
APPENDIX: Figure 6



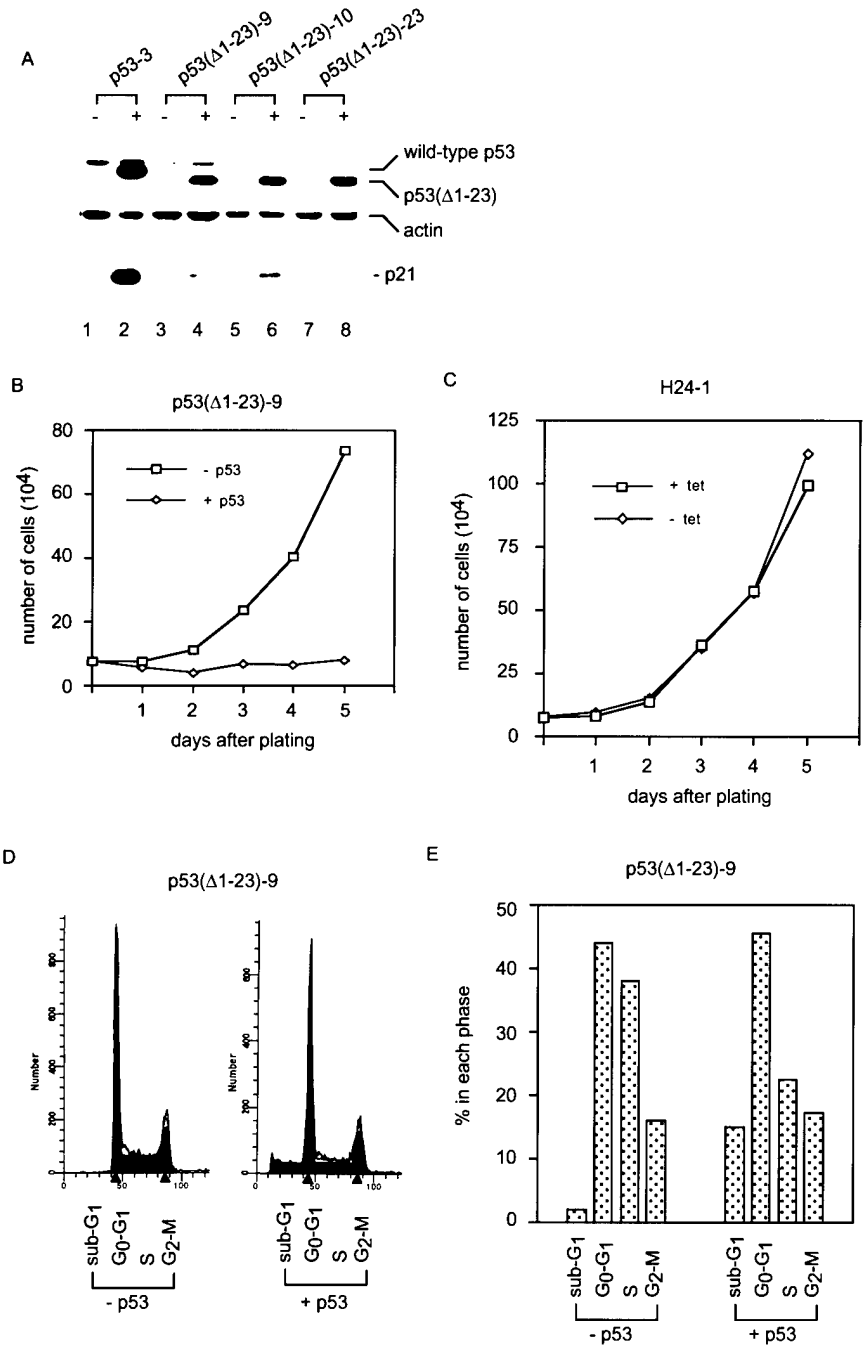
APPENDIX C: Figure 7



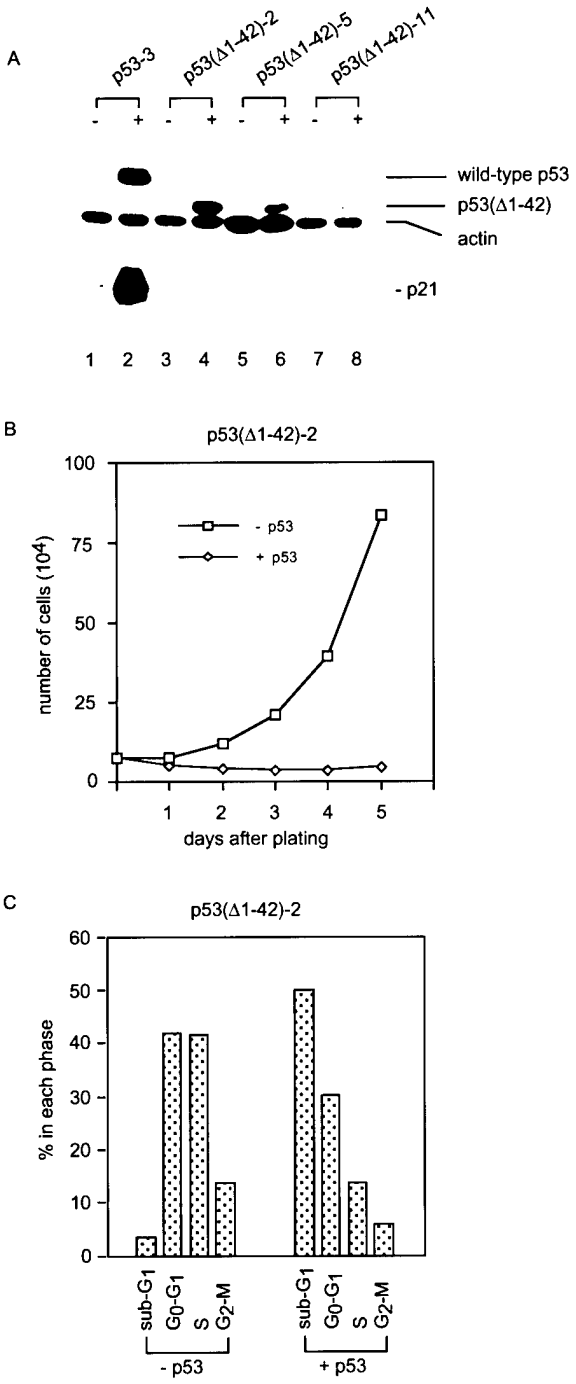




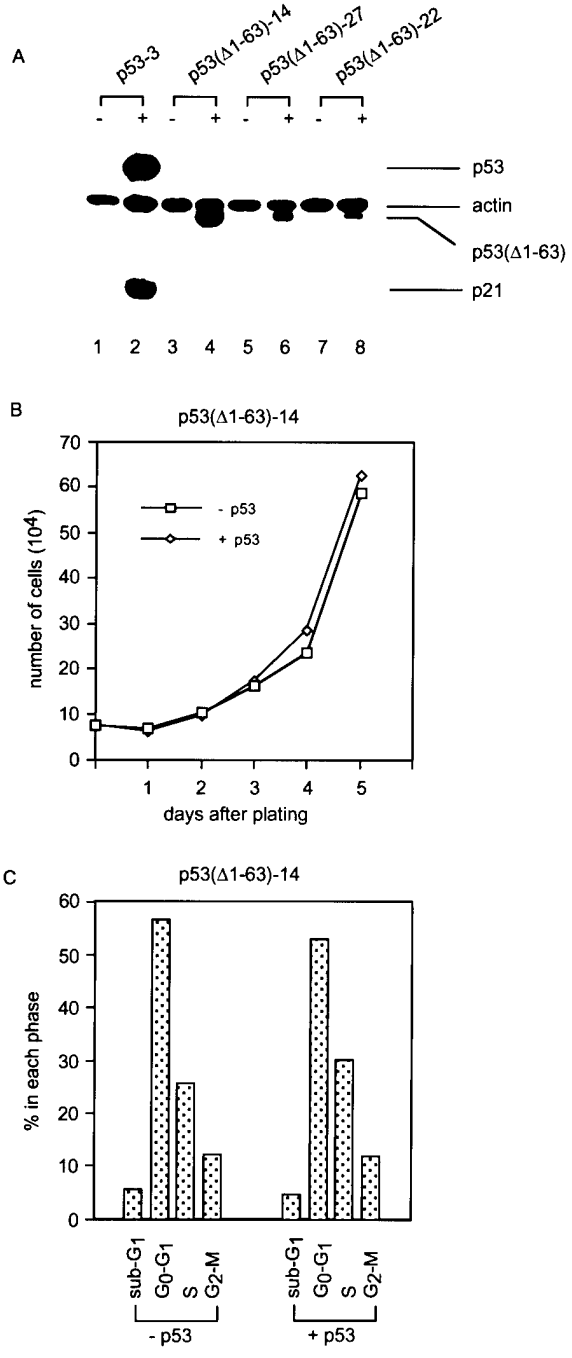
APPENDIX C: Figure 1



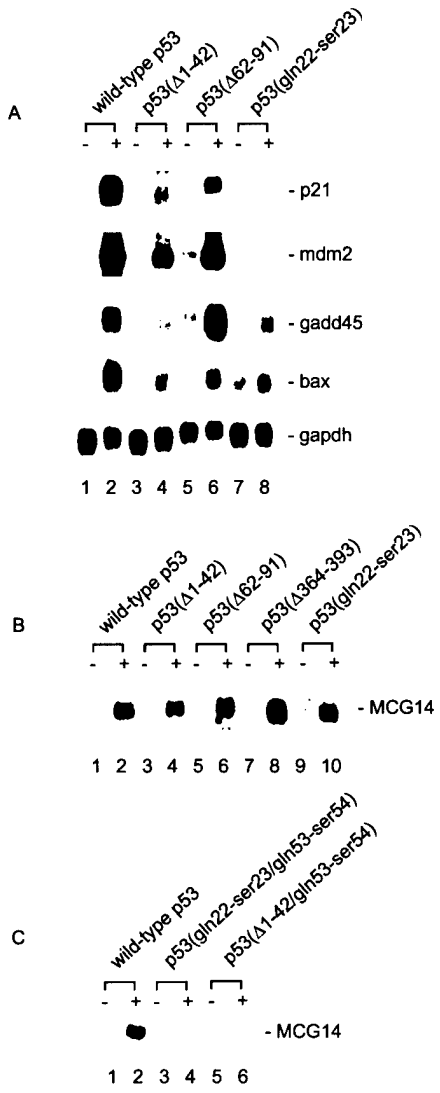
APPENDIX C: Figure 2



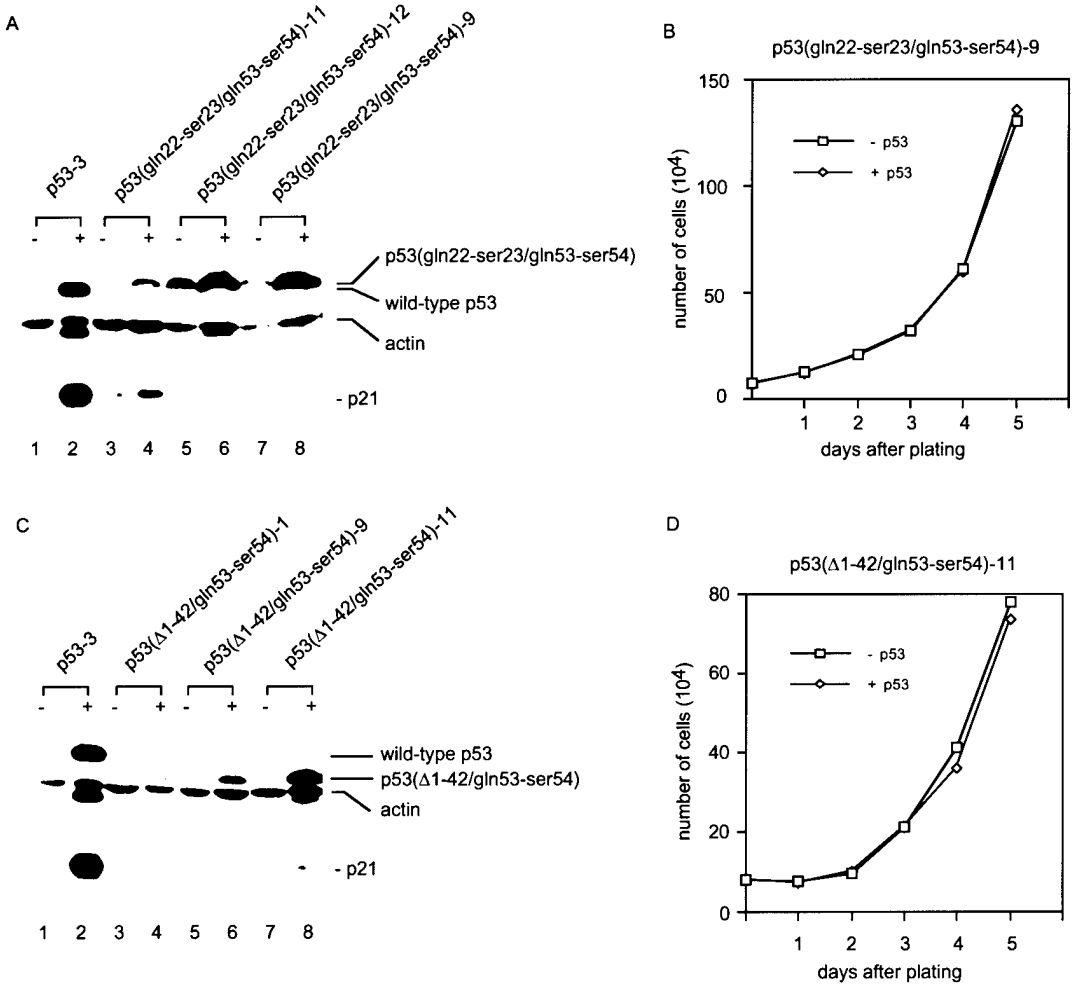
APPENDIX C: Figure 3



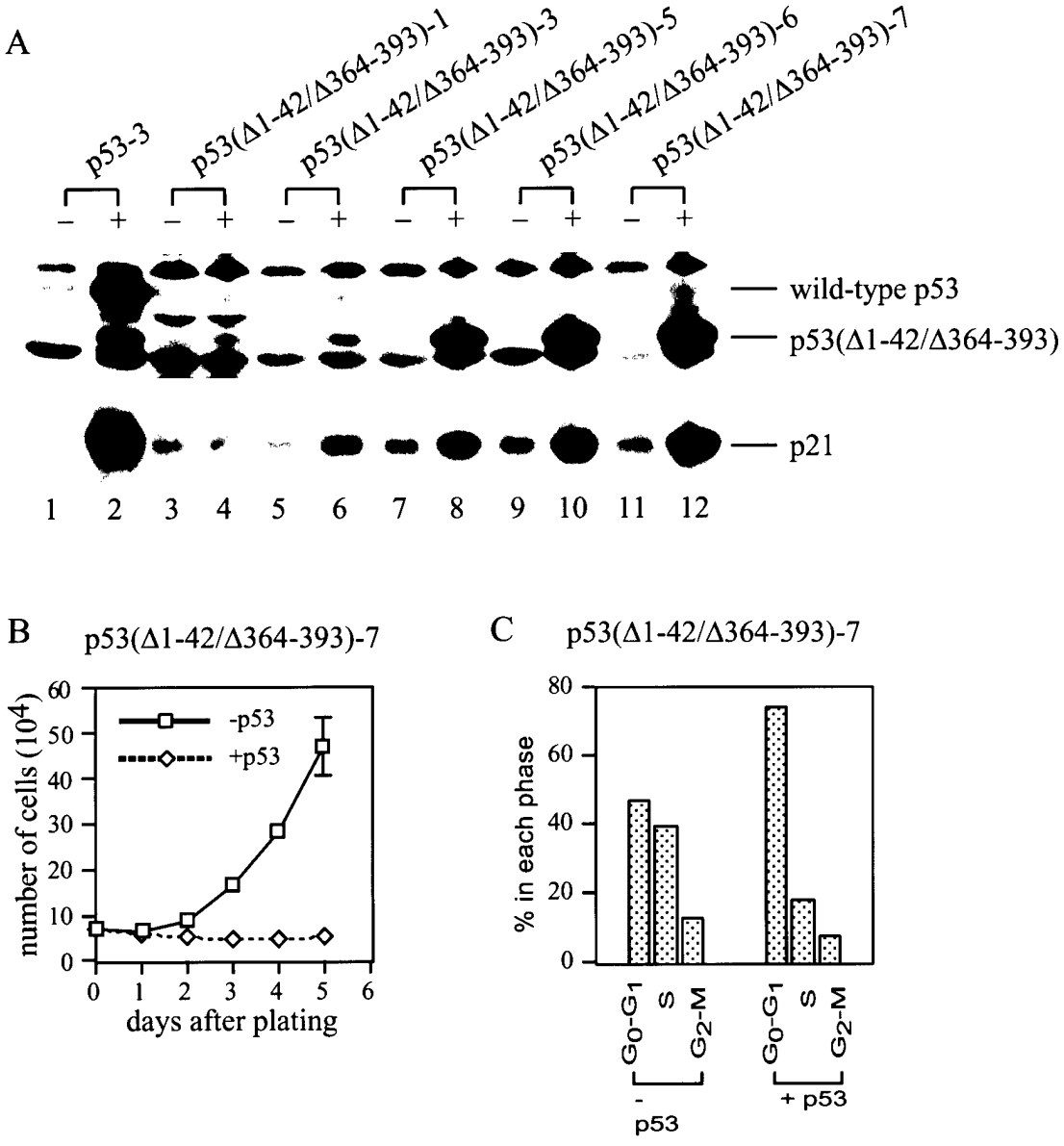
APPENDIX C: Figure 4



APPENDIX C: Figure 5



APPENDIX: Figure 6



APPENDIX C: Figure 7